A method for fractionation of cerebrosides into classes with different fatty acid compositions

AURELIU J. ACHER* and JULIAN N. KANFER

Eunice K. Shriver Center for Research in Mental Retardation, W. E. Fernald State School, Waltham, Massachusetts 02154, and Neurology Research, J. P. Kennedy Jr. Memorial Laboratories, Massachusetts General Hospital, Boston, Massachusetts 02114

Summary A method is described for the separation of beef brain cerebrosides into three fractions containing different classes of fatty acids: nonhydroxy (I), unsaturated nonhydroxy (II), and hydroxy fatty acid cerebrosides (III). The procedure consists of benzoylation of either crude or purified cerebrosides, followed by column chromatographic separation of benzoylated derivatives containing nonhydroxy acids from those containing hydroxy fatty acids. The benzoyl groups are removed by sodium methoxide-catalyzed transesterification; from the reaction mixtures, fractions I and III precipitate. The fraction II present in mother liquor of I was shown to contain mainly short-chain and unsaturated nonhydroxy fatty acid cerebrosides. The fatty acid composition of each fraction was obtained by gas–liquid chromatography.

Supplementary key words cerebrosides · benzoylation · chromatographic separation · hydroxy fatty acid cerebrosides · nonhydroxy fatty acid cerebrosides · unsaturated nonhydroxy fatty acid cerebrosides

CHROMATOGRAPHIC METHODS have provided evidence for the existence of a large number of cerebrosides which differ from each other primarily in their fatty acid components (1). These cerebrosides have been isolated as individual spots on TLC, and their fatty acid compositions have been obtained from GLC peaks (2–6). However, in spite of a large variety of solvent systems employed for the TLC separation of the cerebrosides, their resolution usually remains incomplete because an "individual" spot may yield more than one fatty acid upon hydrolysis.

For the column chromatographic separation of cerebrosides, silicic acid (7–10), Florisil (3, 11), and alumina (12) have been employed as adsorbents. Multiple TLC development (13) or fractionation of the peracetylated components of cerebron fraction by countercurrent distribution (14) has also been used. Klenk and Schorsch (15) have treated the acetylated cerebrosides with mercuric acetate in order to obtain those containing unsaturated fatty acids. A more recent publication indicated the separation of cerebrosides into two classes by a hydroxylapatite column; however, no details concerning the compositions of these materials are provided (16).

The present method takes advantage of the large difference between the R_F values of benzoylated derivatives of cerebrosides containing nonhydroxy and hydroxy fatty acids which facilitates their separation on chromatographic column without overlapping of fractions. The procedure is simple, the yields are nearly quantitative, and the method may be applied to a "crude" sphingolipid preparation.

Experimental procedure

Materials and methods. Beef brains were obtained from a local slaughter house. Reagent grade pyridine (Merck) was distilled from BaO and stored over KOH; benzoyl chloride (Merck) was freshly distilled before use. Redistilled reagent grade methanol (Baker) and dry HCl (Matheson) were employed for preparation of methanolic HCl. Hexamethyldisilazane, trimethylchlorosilane, and the GLC column packings were obtained from Applied Science Laboratories Inc., State College, Pa. GLC of the fatty acids was carried out with a 762A research chromatograph (Hewlett-Packard), using the flame ionization detector. IR spectra were obtained in a Perkin-Elmer model 237 instrument. Melting points were taken on a Fisher-Johns apparatus. All solvents used for both thin-layer and column chromatography were distilled prior to use. Silicic acid (Mallinckrodt Chemical Works), Hyflo Super-Cel (Johns-Manville), and Unisil (Clarkson Chemical Co.) were used for column chromatography. Thin-layer plates precoated with silica gel G (250 μ) were obtained from Analtech, Inc. Free and benzoylated cerebrosides were detected on TLC with a spray consisting of anisaldehyde, concentrated H_2SO_4, and distilled ethanol (1:1:18); fatty acid methyl esters were detected with a solution of formaldehyde (37%) and concentrated H_2SO_4 (3:97). The plates were heated at 140°C for 10 min after spraying.

Isolation of the cerebrosides. The sphingolipids were extracted from bovine brain using the procedure of Folch, Lees, and Sloane Stanley (17). Neutral lipid was removed from this mixture by successive extractions with diethyl ether (9). The crude material was dissolved in a mixture of chloroform and methanol and then freed of phosphoglycerides by sodium methoxide treatment (18). The saponification reaction mixture was neutralized with methyl formate and insoluble materials were removed by filtration and fractioned (17). The upper phases were discarded and the lower layer was dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo. Pure cerebrosides were obtained from this residue by

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography. The term cerebroside is used to designate galactosyl-*N*-acyl-sphingosine.

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silica gel column chromatographic purification (9). Alternatively, a "crude" cerebroside was prepared by two crystallizations from hot methanol. Both of these preparations were used as starting materials for the synthesis of the benzoylated cerebrosides.

Benzoylation of the cerebrosides. 100 ml of pyridine was added to 2 g of either purified or "crude" cerebrosides which had been thoroughly dried in vacuo over P_2O_5 for 48 hr. The suspension was stirred while cooled in an ice-water bath (5–10°C), and 4 ml of freshly distilled benzoyl chloride was added. The reaction flask was stoppered and left at room temperature for 24 hr and the reaction mixture was poured over 300 g of chopped ice containing 100 ml of concentrated HCl. After the ice was melted, 250 ml of $CHCl_3$ was added, the biphasic mixture was transferred to a separatory funnel, and the upper aqueous layer was discarded. The lower layer was washed successively with cold concentrated HCl (60 ml, once), water (100 ml, once), 5% $NaHCO_3$ (100 ml, three times), and finally with water until neutral to pH paper. The $CHCl_3$ solution was dried over Na_2SO_4, filtered, and evaporated to dryness in vacuo. The residue from the benzoylation was dissolved in 15 ml of benzene and applied to a column of silicic acid (200 g, 100 mesh, 60 × 140 mm). After washing with 1.2 liters of benzene, elution was carried out with 1.2 liters of benzene–ethyl acetate 133:1 (first eluate) and then with 1.2 liters of a more polar mixture of the same solvents (50:1, second eluate). The eluates were concentrated to dryness and the yields were 0.92 and 0.4 g (Fig. 1*A*, lane *1*, R_F = 0.71) and 2.3 and 1.1 g from pure and "crude" cerebrosides, respectively (Fig. 1*A*, lane *3*, R_F = 0.38). Both derivatives were obtained as oily materials which resisted crystallization. TLC of the benzoylated cerebrosides was performed in a solvent mixture of benzene–ethyl acetate 40:3. The high ratio of benzoyl chloride to cerebroside used for the formation of these derivatives (14 moles/mole) did not appear to affect the amide group and assured a complete substitution of all available hydroxyl groups. The IR spectra (using NaCl discs) of these derivatives were almost identical, and there was no evidence for the presence of either free hydroxyl groups or diacyl amide formation. The yields of the benzoylated product and the subsequent deesterified materials were almost theoretical.

Preparation of free cerebrosides. The benzoylated product from the first column eluate (R_F = 0.71, 1.0 g) was dissolved in absolute CH_3OH (20 ml) and submitted to

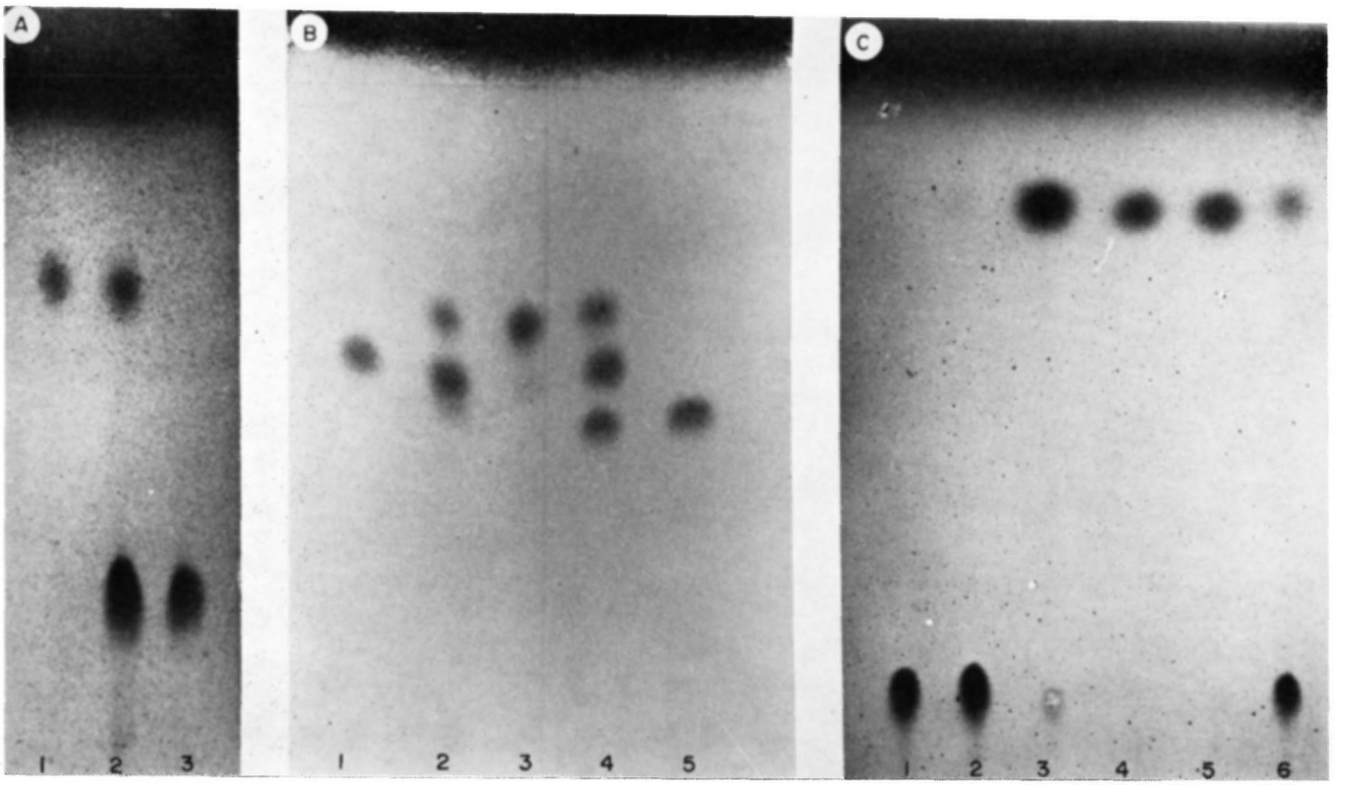

FIG. 1. *A:* TLC of benzoylated pure cerebrosides separated by column chromatography. Lane *1*, benzene–ethyl acetate 133:1 eluate; lane *2*, reaction mixture before column separation; lane *3*, benzene–ethyl acetate 50:1 eluate. *B:* TLC of products obtained after $NaOCH_3$ debenzoylation. Lane *1*, cerebroside fraction III; lane *2*, standard mixture of cerebroside; lane *3*, cerebroside fraction I; lane *4*, mixture of lanes *1*, *3*, and *5* after separation; lane *5*, cerebroside fraction II. *C:* TLC of methyl esters derived from different cerebroside fractions. Lane *1*, fraction III; lane *2*, standard methyl-2-hydroxypalmitate; lane *3*, fraction I; lane *4*, standard methyl palmitate; lane *5*, fraction II; lane *6*, standard mixture of purified cerebroside.

catalytic deacylation by treating it with 1 N sodium methoxide in CH_3OH (0.4 ml). After stirring for 4 hr at room temperature, the reaction mixture was left at 4°C overnight. The precipitate which formed was filtered, washed with cold CH_3OH (3 ml, 3×), and crystallized from hot CH_3OH (8 ml) in a conical test tube. The crystals which formed were harvested by centrifugation (3000 rpm, 20 min, 2°C) and dried in vacuo after decanting the supernatant. The yield of cerebroside fraction I was 0.25 g; R_F = 0.63 (Fig. 1*B*, lane *3*); mp 126–130°C.

The filtrate of cerebroside fraction I was neutralized by passage through a column of Dowex 50W (X8, H^+ form) ion exchange resin, and concentrated in vacuo to dryness. The residue (0.33 g) was purified on a chromatographic column of silicic acid (20 g) and Hyflo Super-Cel (10 g) in $CHCl_3$, from which cerebroside was eluted with a mixture of $CHCl_3$–CH_3OH 11:1 (400 ml). The yield of purified cerebroside fraction II was 0.27 g; R_F = 0.52 (Fig. 1*B*, lane *5*). This material is quite soluble in most organic solvents and resisted all attempts at crystallization.

The second column eluate which contained another benzoylated product (R_F = 0.38, 2.0 g) was dissolved in absolute CH_3OH (50 ml) and treated with sodium methoxide (0.8 ml) in the same manner as above. The precipitate formed during transesterification was filtered, washed with cold CH_3OH (5 ml, 3×) and crystallized from hot CH_3OH (35 ml). The yield of cerebroside fraction III was 1.06 g; R_F = 0.58 (Fig. 1*B*, lane *1*); mp 153–157°C. The IR spectra of the above free cerebrosides were obtained from KBr pellets and were a most identical; TLC was performed in $CHCl_3$–CH_3OH–pyridine–H_2O 40:10:1:1.

Fatty acid compositions of different cerebroside fractions. 3-mg samples of each cerebroside fraction were treated with 1 ml of methanolic HCl (5% w/v) at 70°C for 24 hr in small hydrolysis tubes, as described by Kishimoto and Radin (19). After cooling, the tubes were opened and the solutions were extracted with hexane (1 ml, 3×). The hexane extracts were concentrated to a small volume and transferred to separate columns containing 100 mg of Unisil. The columns were washed with 6 ml of hexane and eluted with 6 ml of benzene. The eluates were evaporated to dryness with a nitrogen stream and the residues were dissolved in 60 μl of $CHCl_3$. TLC of these solutions was performed in hexane–ether 85:15.

The hydroxy fatty acid methyl ester fraction was dried in vacuo and treated with 0.2 ml of a mixture of trimethylchlorosilane and hexamethyldisilazane in pyridine (1:2:5) (20). The mixture was kept at room temperature for 10–15 min and then was heated to 60°C for a few minutes. 2 ml of $CHCl_3$ was added, the mixture was washed with cold H_2O (1 ml, 4×), and the upper phases were discarded. The clear lower phase was evaporated to dryness at room temperature with a nitrogen stream, and the residue was dissolved in 60 μl of $CHCl_3$. Samples (1 μl) from the nonhydroxy fatty acid methyl esters and the trimethylsilyl derivatives of the hydroxy fatty acid methyl esters were injected into the GLC columns. The GLC analyses were carried out at 175°C and each sample was assayed in duplicate using two different columns (3 mm I.D. × 6 ft). One column contained 25% polydiethylene glycol succinate (DEGS, 25%) coated on Chromosorb W (HP, AW, DMCS, 80–100 mesh), and the second contained 3% OV-1, also coated on Chromosorb W. The DEGS column was used isothermally, while the 3% OV-1 column was programmed from 175 to 240°C at a rate of 1°C/min. Helium carrier gas was employed at a flow rate of 70 ml/min.

Results and discussion

Separation of cerebroside fractions I, II, and III. Benzoylation of either the "crude" or purified cerebrosides led to formation of two major products which were separated by silicic acid column chromatography (Fig. 1*A*, lanes *1* and *3*). Three different cerebroside fractions were obtained by catalytic deacylation of these benzoylated derivatives (Fig. 1*B*, lanes *1*, *3*, and *5*). Two of these cerebroside fractions (I and II) were present as benzoyl derivatives (R_F = 0.71) in the first eluate from the column. Cerebroside fraction I was precipitated from the transesterification reaction mixture while II remained in solution. Cerebroside fraction III was obtained from the saponification reaction mixture of the benzoylated derivative (R_F = 0.38) present in the second eluate.

Fatty acid composition of the different fractions. The fatty acid methyl esters formed after methanolysis of the cerebroside fractions (I, II, III) were characterized by TLC and GLC. TLC analysis of fatty acid methyl esters indicated that fractions I and II contained principally nonhydroxy fatty acids (Fig. 1*C*, lanes *3* and *5*). The fatty acids released from fraction III were solely hydroxy fatty acids (Fig. 1*C*, lane *1*).

The nonhydroxy fatty acid methyl esters and the trimethylsily derivatives of hydroxy fatty acid methyl esters were analyzed by GLC for identification and quantification of the individual fatty acids. Two different columns were used in these determinations in order to eliminate false peaks which occasionally appeared in the samples. Comparison of the retention times of the fatty acid derivatives from the different cerebroside fractions on DEGS columns (plotted semilogarithmically) indicated that primarily monoenoic and short-chain fatty acids were present in the cerebrosides of fraction II. The fatty acids were quantitated by measuring the peak areas with an electronic integrator (3370 A, Hewlett-Packard), and the results are given in Table 1. Each

TABLE 1. Fatty acid composition in different cerebroside fractions[a]

Fatty Acid	Fraction I	Fraction II	Fraction III
		%	
14:0[b]		0.3	
16:1		0.6	
16:0	0.3	1.4	0.2
18:1		0.8	
18:0	4.2	21.1	19.6
20:1		0.2	0.8
20:0	0.4	1.0	0.3
22:1		0.6	
22:0	5.5	2.5	4.5
23:1	0.2	1.0	0.3
23:0	10.2	1.7	8.1
24:1	7.5	39.6	7.8
24:0	45.9		32.9
25:1	2.9	12.2	2.5
25:0	12.7		10.4
26:1	4.4	14.9	6.5
26:0	4.7		3.5
27:1	0.6	1.3	1.2
27:0	0.1		0.7
28:1	0.3	0.8	0.4
28:0			0.2

[a] Fatty acids present in amounts below 0.1% were omitted. The fatty acid composition of starting purified cerebrosides was that anticipated from the individual fractions.

[b] Number of carbon atoms: number of double bonds (e.g., 18:0 = stearic acid).

sample of methyl esters was analyzed by GLC at least twice. Three or four separations of cerebrosides were made, and the analyses were always almost identical. The data in Table 1 are from one representative separation.

From a practical point of view, our method allows the separation of the cerebrosides containing hydroxy fatty acids from those containing nonhydroxy fatty acids. The separation based on unsaturation is not quantitative, since fractions I and II still contain unsaturated (16%) and saturated (28%) fatty acids, respectively. A major advantage of this method is the ability to obtain gram quantities of these compounds.

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